Gene panel sequencing identifies a likely monogenic cause in 7% of 235 Pakistani families with nephrolithiasis

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Abstract: Nephrolithiasis (NL) affects 1 in 11 individuals worldwide and causes significant patient morbidity. We previously demonstrated a genetic cause of NL can be identified in 11-29% of pre-dominantly American and European stone formers. Pakistan, which resides within the Afro-Asian stone belt, has a high prevalence of nephrolithiasis (12%) as well as high rate of consanguinity (> 50%). We recruited 235 Pakistani subjects hospitalized for nephrolithiasis from five tertiary hospitals in the Punjab province of Pakistan. Subjects were surveyed for age of onset, NL recurrence, and family history. We conducted high-throughput exon sequencing of 30 NL disease genes and variant analysis to identify monogenic causative mutations in each subject. We detected likely causative mutations in 4 of 30 disease genes, yielding a likely molecular diagnosis in 7% (17 of 235) of NL families. Only 1 of 17 causative mutations was identified in an autosomal recessive disease gene. 10 of the 12 detected mutations were novel mutations (83%). SLC34A1 was most frequently mutated (12 of 17 solved families). We observed a higher frequency of causative mutations in subjects with a positive NL family history (13/109, 12%) versus those with a negative family history (4/120, 3%). Five missense SLC34A1 variants identified through genetic analysis demonstrated defective phosphate transport. We examined the monogenic causes of NL in a novel geographic cohort and most frequently identified dominant mutations in the sodium-phosphate transporter SLC34A1 with functional validation.

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Abstract

Nephrolithiasis (NL) affects 1 in 11 individuals worldwide and causes significant patient morbidity. We previously demonstrated a genetic cause of NL can be identified in 11–29% of pre-dominantly American and European stone formers. Pakistan, which resides within the Afro-Asian stone belt, has a high prevalence of nephrolithiasis (12%) as well as high rate of consanguinity (> 50%). We recruited 235 Pakistani subjects hospitalized for nephrolithiasis from five tertiary hospitals in the Punjab province of Pakistan. Subjects were surveyed for age of onset, NL recurrence, and family history. We conducted high-throughput exon sequencing of 30 NL disease genes and variant analysis to identify monogenic causative mutations in each subject. We detected likely causative mutations in 4 of 30 disease genes, yielding a likely molecular diagnosis in 7% (17 of 235) of NL families. Only 1 of 17 causative mutations was identified in an autosomal recessive disease gene. 10 of the 12 detected mutations were novel mutations (83%). *SLC34A1* was most frequently mutated (12 of 17 solved families). We observed a higher frequency of causative mutations in subjects with a positive NL family history (13/109, 12%) versus those with a negative family history (4/120, 3%). Five missense *SLC34A1* variants identified through genetic analysis demonstrated defective phosphate transport. We examined the monogenic causes of NL in a novel geographic cohort and most frequently identified dominant mutations in the sodium–phosphate transporter *SLC34A1* with functional validation.

Introduction

Nephrolithiasis (NL) affects 1 in 11 individuals during their lifetime (Scales et al. 2012; Tasian et al. 2015, 2016). NL is associated with significant patient morbidity, recurrence, and healthcare costs (Germino and Kirkali 2015; Rule et al.

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2009). The causes of NL are not well understood. Until recently, monogenic causes of NL were thought to be limited to rare tubulopathies and genetic syndromes. However, we have established that a single-gene cause of NL can be identifi in 11% and 16.7–29.4% of selected adult and pediatric stone formers, respectively (Braun et al. 2016a; Daga et al. 2017; Halbritter et al. 2015). These studies were comprised of predominantly American and European stone formers and, therefore, the broader relevance of these findings worldwide is not clear.

Pakistan resides within the "Afro-Asian stone belt", a region with a high prevalence of nephrolithiasis (12%) (Rizvi et al. 2002). Environmental risk factors, including chronic dehydration and nutrition, contribute to these higher rates of nephrolithiasis (Rizvi et al. 2002). Pakistan also has a high rate of consanguineous marriages (> 50%) (Romeo and Bittles 2014), which by nature of Mendelian genetics is associated with an increased prevalence of autosomal recessive genetic diseases (Overall et al. 2003). We hypothesized that NL disease gene mutations cause nephrolithiasis in a significant fraction of Pakistani stone formers. However, the prevalence of monogenic forms of nephrolithiasis in this population is unknown.

We, therefore, performed high-throughput gene panel sequencing analysis of 30 known NL-causing genes in a cohort of 235 Pakistani subjects with nephrolithiasis recruited from 1 of 5 different tertiary care hospitals in Punjab, Pakistan. We show that likely causative mutations in known NL-causing genes are present in 7% of stone formers. We furthermore provide practical therapeutic and preventative measures based on each molecular diagnosis.

Materials and methods

Human subjects

Subjects were recruited during admission for nephrolithiasis, which was confirmed by abdominal ultrasound. The subjects were admitted to one of five diff rent tertiary care hospitals in Punjab, Pakistan from July 2014 to December 2016 (Suppl. Figure 1). Subjects received informed consent and provided clinical information (confirmed by their urologist and/or medical records), family history for pedigree construction, and a blood sample for DNA extraction. Consanguinity was determined by history from adult subjects and/or guardians for pediatric cases by asking if parents of subjects were related. Ten subjects did not consent to the study, and seven subjects were excluded as they had an evident secondary cause of NL (including urinary tract infections and secondary hyperoxaluria). Of the remaining 242 families, adequate DNA samples for genetic studies were obtained in 235 (Suppl. Figure 1). For 31 families, additional family members were recruited upon consent for clinical information and DNA submission, allowing for multi-generational pedigree construction. Serum chemistries, urine metabolites and stone analyses were requested and obtained when available. In total, the cohort consisted of 440 individuals (235 initial probands, 115 additional affected family members and 90 unaffected family members) in 235 families. The study was approved by the institutional review board of Boston Children's Hospital and the Ethical Review Committee for Medical and Biomedical Research, University of Health Sciences, Lahore, Pakistan. It adheres to the Declaration of Helsinki.

Gene panel sequencing

We performed mutation analysis using barcoded multiplex PCR (Fluidigm 48.48-Access Arrays™)-based approach that we developed previously (Braun et al. 2016a; Halbritter et al. 2012, 2013, 2015). We designed 518 target-specifi primers for 381 coding exons and the adjacent splice sites of 30 genes that are known monogenic causes of nephrolithiasis or nephrocalcinosis (defined by an OMIM phenotype). The genes screened included *AGXT, APRT, ATP6V0A4, ATP6V1B1, CA2, CASR, CLCN5, CLCNKB, CLDN16, CLDN19, CYP24A1, FAM20A, GRHPR, HNF4A, HOGA1, HPRT1, KCNJ1, OCRL, SLC12A1, SLC22A12, SLC2A9, SLC26A1, SLC34A1, SLC34A3, SLC3A1, SLC4A1, SLC7A9, SLC9A3R1, VDR*, and *XDH* (Suppl. Table 1). Amplicon sizes were chosen to range from 250 to 300 bp. Primer sequences will be provided upon request. The use of barcoded multiplex PCR (Fluidigm 48.48-Access Arrays™ system) allowed parallel amplification of all 518 amplicons in 48 patients at a time. Subsequently, the pooled libraries were sequenced on an Illumina MiSeq® instrument. Sequence reads were aligned to the human reference sequence using CLC Genomics Workbench™ (CLC-bio, Aarhus, Denmark) (Braun et al. 2016a; Halbritter et al. 2012, 2013, 2015) to identify variants that were distinct from the reference human genome (Suppl. Figure 2 and Suppl. Figure 3). We aimed for minimum 20 × coverage of 80% of exons in each sample and repeated sequencing of samples that did not meet this initial standard.

Mutational analysis

We excluded synonymous variants and variants that occur with minor allele frequencies > 1% in the dbSNP (version 147) database (Suppl. Figure 2 and Suppl. Figure 3). Variant frequency was further assessed in exome and genome databases EVS, ExAC, and gnomAD of healthy control individuals. Remaining variants were then evaluated as in Suppl. Figure 2 and Suppl. Figure 3. All remaining variants were confi med in original subject DNA by Sanger

sequencing with segregation in family members when DNA was available.

Statistical testing

Odds ratios were measured using the online resource at MedCalc (see "Web resources").

Expression of *SLC34A1* wildtype and mutant RNA in *Xenopus laevis* oocytes

Animal studies adhere to the NIH guide for the Care and Use of Laboratory Animals or the equivalent. *Xenopus laevis* oocytes were purchased from EcoCyte and transferred to the standard extracellular solution for oocyte experiments containing 100 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 10 mM HEPES, pH 7.4 adjusted with Tris. Inorganic phosphate was added from a 1M K_2HPO_4/ KH_2PO_4 stock premixed to give pH 7.4. Modified Barth's solution for storing oocytes contained (in mM) 88 NaCl, 1 KCl, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 2.5 $NaHCO_3$, 2 $Ca(NO_3)_2$, 7.5 HEPES, pH 7.5 adjusted with Tris and supplemented with 5 mg/l doxycyclin and 5 mg/l gentamicin. All standard reagents were obtained from either Sigma-Aldrich or Fluka (Buchs, Switzerland). Oocytes were injected with 50 nl of cRNA (200 ηg/µl for single injection, 100 ng/µl total RNA for coinjection) of wildtype or mutant *SLC34A1* constructs or water as negative control (NI). Constructs were designed as discussed in Supplementary Method 1. Experiments were performed 3 days after injection.

^{32}Phosphate uptake

Water-injected control oocytes (NI), oocytes expressing wildtype and mutant *SLC34A1* (6–8 oocytes/group) were first allowed to equilibrate in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.4 adjusted with Tris) without tracer. After aspiration of this solution, oocytes were incubated in ND96 solution containing 1 mM cold P_i and $^{32}P_i$ (specific activity 10 mCi $mmol^{-1}$ Pi, PerkinElmer). Uptake proceeded for 10 min, then oocytes were washed four times with ice-cold ND96 and lysed individually in 2% SDS for 30 min before the addition of the scintillation cocktail (Emulsifier-Safe, PerkinElmer). The amount of radioactivity in each oocyte was measured by scintillation counting (Tri-Carb 29000TR, Packard).

Webresources

UCSC Genome Browser, https://genome.ucsc.edu.

Exome Variant Server, http://evs.gs.washington.edu/EVS.

Exome Aggregation Consortium, http://exac.broadinstitute.org.

Genome Aggregation Database, http://gnomad.broadinstitute.org/.

Online Mendelian Inheritance in Man (OMIM), http://www.omim.org.

Polyphen2, http://genetics.bwh.harvard.edu/pph2.

Sorting Intolerant from Tolerant (SIFT), http://sift.jcvi.org.

MutationTaster, http://www.mutationtaster.org.

MedCalc Odds Ratio Calculator, https://www.medcalc.org/calc/odds_ratio.php.

Results

Cohort characteristics

We recruited a diverse cohort of stone formers across sex, age, nephrolithiasis history, and familial inheritance, as evident from demographic data of probands from these families. 143 probands were male and 92 were female (M:F ratio 1.6:1). Further clinical data were available for 229 of 235 probands. The age of first NL episode spanned pediatric and adult age groups: 1–20 years, 74/229 (32%); 21–40 years, 108/229 (47%); 41–60 years, 42/229 (18%); 61–80 years, 5/229 (2%). The median age of onset was 28 years (range 1–76 years). 113 (49%) of 229 probands reported a history of recurrent stones. 47% (109/229) of probands had a positive family history of NL. 53% (122/229) were born from consanguineous unions.

Monogenic causative mutation detection

Using high-throughput exon sequencing of 30 NL disease genes in 235 subjects with renal stones, we identifi a molecular diagnosis in 7% (17 of 235 NL families) in 4 different known NL genes *SLC34A1, SLC4A1, SLC7A9*, and *APRT* (Fig. 1). Panel sequencing results were confirmed by Sanger sequencing (Suppl. Figures 4 and 5). Heterozygous mutations were detected in the 3 dominant genes *SLC34A1* (12 families), *SLC4A1* (3 families), *SLC7A9* (1 family), while biallelic mutations were identified only in 1 recessive gene *APRT* (1 family) as described in Table 1 and Fig. 1. 10 of 12 mutations (83%) were novel. Mutations in *SLC34A1* were identified in two families UF26 and UF30 where additional family members with stones were recruited, and segregation was confirmed (Suppl. Figure 4).

Genotype relationships

We next evaluated whether molecular diagnoses correlated with geographic location (i.e., founder eff and specific clinical features. First to assess for founder mutations, genetic mutations were mapped according to the specifi

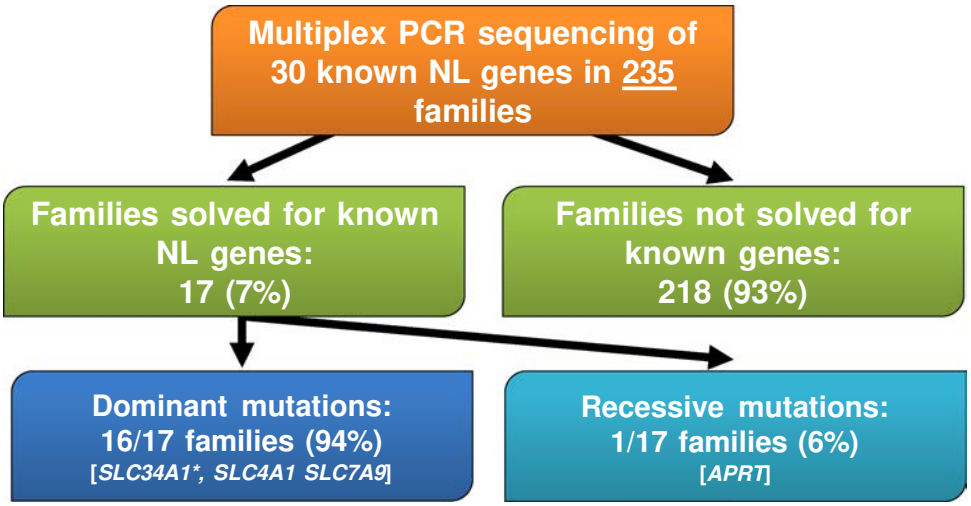

Fig. 1 Fraction (%) of 235 Pakistani NL families in whom a causative mutation was detected in 1 of 30 known NL genes ("solve rate"). The flow diagram depicts the distribution of NL families by solve rate and by mode of inheritance (recessive versus dominant). *NL* nephrolithiasis. "Asterisk" indicates mutations in *SLC34A1* determined based on standardized genetic analysis and functional analysis (in case of missense mutations)

town or city where each subject lived (Fig. 2). Mutations were diffusely distributed through Punjab and surrounding provinces in Pakistan. No *SLC34A1* mutations that were detected in multiple families clustered within specific towns or cities, suggesting no founder effects in our cohort.

We next assessed for relationships between the age of stone onset and molecular diagnosis. To begin, we plotted frequency of mutation detection *versus* age of onset, observing that mutation detection frequency increases with earlier age of onset (Fig. 3): 1–20 years, 8 of 74 (11%); 21–40 years, 7 of 108 (7%); 41–60 years, 2 of 42 (5%); 61–80 years, 0 of 5 (0%).

Finally, we compared mutation detection frequency to history of stone recurrence, family history of stone disease, and parental consanguinity (Suppl. Table 2). We observed a higher frequency of mutation detection in subjects with a positive NL family history (13/109, 12%) versus those with a negative family history (4/120, 3%) that was statistically significant (Suppl. Table 2). There were no correlations between the frequency of mutation detection and stone recurrence or consanguinity status. These analyses indicate that certain clinical factors such as earlier age of onset and family history were associated with an increased likelihood of identifying a molecular diagnosis.

SLC34A1 mutations

As *SLC34A1* mutations were identifi in 12 families, we compared age of onset with severity of mutation (Suppl. Figure 6) but did not detect a clear genotype–phenotype relationship.

Five of seven *SLC34A1* mutations that we identified are novel, including four of five missense mutations (Table 1). *SLC34A1* encodes the sodium–phosphate co-transporter NaPiIIa. We evaluated the impact of these mutations on NaPiIIa cell surface localization and phosphate transport. When co-expressed with wildtype *SLC34A1*, all missense mutant constructs localized appropriately on the cell surface by confocal microscopy (Suppl. Figure 7). When mutated *SLC34A1* RNA was expressed in *Xenopus* oocytes, the fi e novel missense mutations showed defective phosphate uptake in isolation (Fig. 4). An additional variant of unknown significance (c.365G>C; p.Ser122Thr) was identified in family URO131 (Suppl. Table 3) but did not impair transporter localization or phosphate uptake (Fig. 4 and Suppl. Figure 7). To assess for dominant negative eff of our mutations, mutant *SLC34A1* RNA was co-injected in oocytes with wildtype RNA at equimolar ratios. No mutant constructs suppressed phosphate transport below the activity of oocytes expressing 50% of wildtype *SLC34A1* RNA, suggesting these mutations do not have dominant negative effects on wildtype NaPiIIa.

Discussion

Nephrolithiasis is a medical condition that afflicts patients worldwide, requiring invasive interventions and hospitalizations, precipitating kidney injury, and leading to growing healthcare costs (Germino and Kirkali 2015; Scales et al. 2012). We have previously demonstrated that a single-gene cause can be identifi in 11–29% of cases depending on age of onset (Braun et al. 2016a; Daga et al. 2017; Halbritter et al. 2015). However, these previous fi were based on cohorts primarily recruited from the United States and Europe.

Here, we performed mutational analysis in a large cohort of Pakistani stone formers with nephrolithiasis at presentation and provide the first large-scale evaluation of monogenic stone disease in the Afro-Asian stone belt. We sequenced the coding regions of 30 established NL disease genes and identified the likely causative mutation in 17 of

Table 1 Molecular genetic diagnoses established in 3 dominant genes in 20 individuals from 16 NL families (6.8%) and in 1 recessive gene in 1 family (0.4%) out of 235 families with NL from Punjab province in Pakistan

Family_Individual	Nucleotide change	Amino acid change	Zygosity state	PPh2, evolutionary conservation	gnomAD allele frequency[a]	Ref.	Sex	Age of onset (years)	Stone recurrence	Family history	Parental consanguinity	Phenotype prior to mutational analysis
Dominant genes												
SLC34A1 (type 2 sodium–phosphate cotransporter)												
UF26-2	c.511G>A	p.Val171Ile	het	0.881, *D r.*	1/37/276,942	Novel	F	35	Yes	Y	Y	NL
UF26-3	c.511G>A	p.Val171Ile	het	0.881, *D r.*	1/37/276,942	Novel	M	30	No			NL
UF26-4	c.511G>A	p.Val171Ile	het	0.881, *D r.*	1/37/276,942	Novel	M	20	Yes			NL
UF30-3	c.653C>T	p.Ala218Val	het	0.899, *C i.*	0/23/276,526	Novel	M	22	No	Y	Y	CaOx NL
UF30-4	c.653C>T	p.Ala218Val	het	0.899, *C i.*	0/23/276,526	Novel	F	30	No			NL
URO-11	c.652G>A	p.Ala218Thr	het	0.784, *C i.*	2/100/276,574	Novel	M	25	Yes	Y	Y	NL
URO-12	c.398C>T	p.Ala133Val	het	0.991, *C.i.*	3/1003/277,188	Y	F	55	Yes	N	N	NL
URO-13	c.1225G>A	p.Gly409Ser	het	0.999, *C.e.*	0/27/246,234	Novel	M	17	No	Y	Y	NL
URO-17	c.293dup	p.Ala99Argfs*37	het	NA	NP	Novel	M	18	No	N	Y	NL
URO-29	c.398C>T	p.Ala133Val	het	0.991, *C.i.*	3/1003/277,188	Y	M	40	Yes	Y	Y	NL
URO-88	c.293dup	p.Ala99Argfs*37	het	NA	NP	Novel	M	40	Yes	N	N	NL
URO-117	c.1006+1G>A	Splice site	het	NA	0/16/246,190	Y	F	17	No	Y	Y	NL
URO-133	c.398C>T	p.Ala133Val	het	0.991, *C.i.*	3/1003/277,188	Y	M	8	Yes	N	Y	CaOx NL
URO-140	c.1006+1G>A	Splice site	het	NA	0/16/246,190	Y	M	36	Yes	Y	N	NL
URO-169	c.398C>T	p.Ala133Val	het	0.991, *C.i.*	3/1003/277,188	Y	M	52	Yes	Y	N	NL
SLC4A1 [anion exchanger (Diego blood group)]												
URO-35	c.286C>T	p.Arg96Cys	het	0.838, *M.m.*	0/73/246,164	Novel	F	20	No	Y	N	NL
URO-178	c.911G>A	p.Arg304Gln	het	0.752, *X.t*	0/23/237,418	Novel	M	1	No	N	Y	NL
URO-196	c.1541G>C	p.Arg514Pro	het	0.948, *C.i.*	0/1/30,782	Novel	F	40	No	Y	N	NL
SLC7A9 [solute carrier family 7 (glycoprotein-associated amino acid transporter light chain, bo,+ system), member 9]												
URO-152	c.1076_1077dup	p.Ile360Valfs*3	het	NA	NP	Novel	M	24	No	Y	N	NL
Recessive gene												
APRT (adenine phosphoribosyltransferase)												
URO-77	c.292_293del	p.Trp98Glyfs*11	Hom	NA	NP	Novel	M	3	No	Y	Y	NL

CaOx calcium oxalate; *C.e. Caenorhabditis elegans; D.r. Danio rerio; F* female; *het* heterozygous; *Hom* homozygous; *M* male; *M.m. Mus musculus; N* no; *NA* not applicable; *ND* No data; *NL* nephrolithiasis; *NP* not present; *PPh2* Polyphen2-HumVar (http://genetics.bwh.harvard.edu/pph2/); *X.t. Xenopus tropicalis; Y* yes

[a]Frequency reported as "number of homozygotes/number of variant alleles/total alleles" in Genome Aggregation Database (gnomAD)

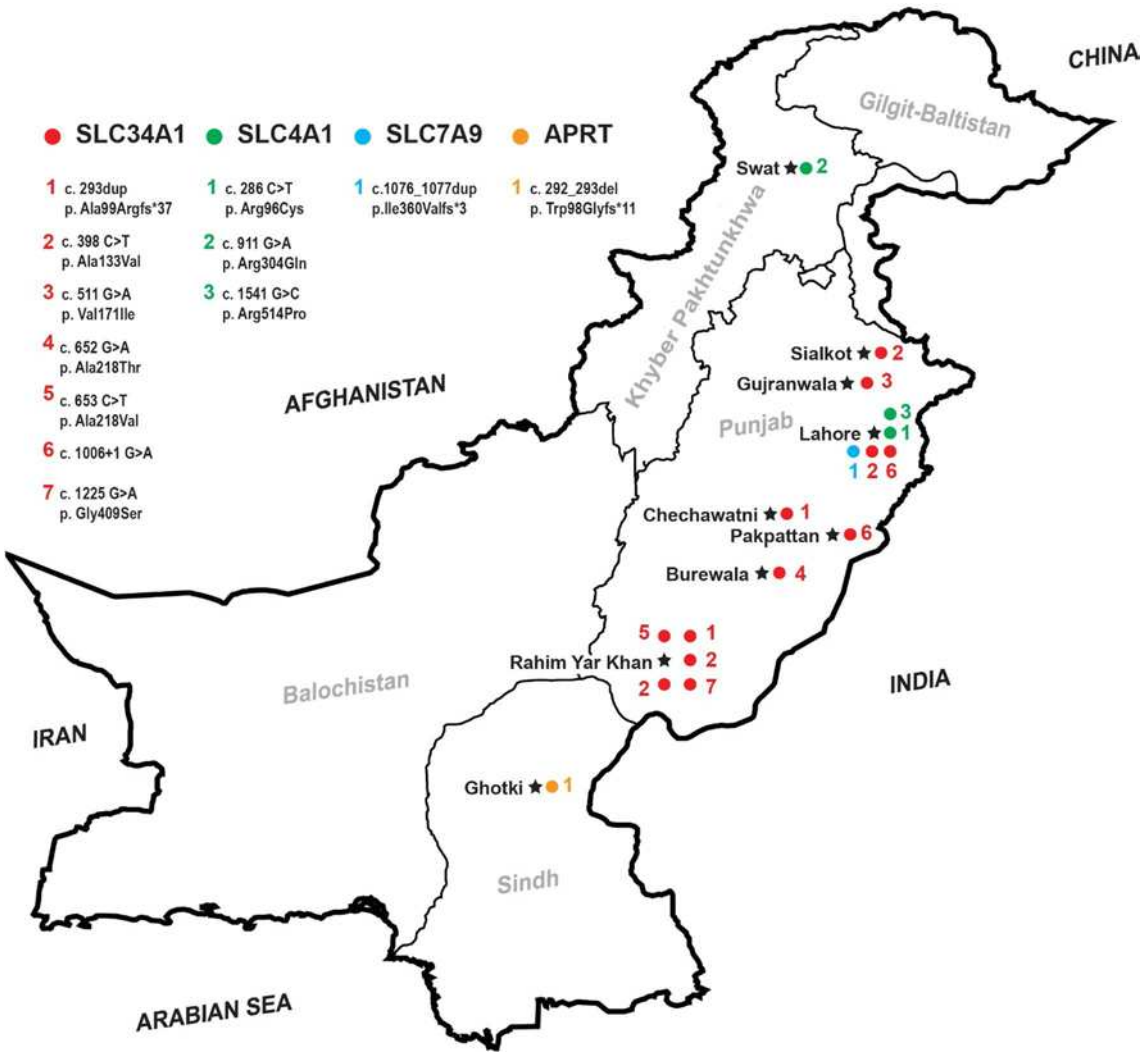

Fig. 2 Regional distribution of 12 different mutations in 4 known NL genes (*SLC34A1, SLC4A1, SLC7A9, APRT*) detected in NL families in Punjab and 2 neighboring provinces of Pakistan. The *SLC34A1* mutations p.Ala99Argfs*37, c.1006+1G>A, p.Ala218Val and p.Ala133Val were observed in multiple families (2, 2, 2, and 4, respectively) but did not aggregate within sub-regions of Pakistan, suggesting these are not founder alleles

235 (7%). This study confirms our previous findings (Braun et al. 2016a; Daga et al. 2017; Halbritter et al. 2015) that mutations in NL disease genes can explain a substantial fraction of disease in stone forming individuals.

We detected a causative mutation more frequently in those with a positive family history of NL than those lacking a family history of NL (Suppl. Table 2), which was reported in renal stone disease previously (Halbritter et al. 2015; Stechman Michael et al. 2007). This finding is consistent with a vertical pattern of inheritance, which we would expect from the mainly dominant mutations identified in our cohort. We also identified a causative mutation more frequently in those with early age of onset relative to those with later age of onset, consistent with previous studies (Braun et al. 2016a; Daga et al. 2017; Halbritter et al. 2015). In future studies, it will be important to consider these genetic diagnoses in correlation with additional clinical presenting factors and biomarkers that impact patient management including stone analysis, serum chemistries and urine metabolite studies.

The frequency of causative mutations in Pakistani stone formers overall was lower than previously observed in Halbritter et al. 2015 (7.2% here versus 15%). Both studies had similar percentages of pediatric cases (32% in this study versus 36%), which should not, therefore, bias mutation detection. Diff rences in the genetic landscape of Pakistani stone formers versus other populations may also contribute. Moreover, “unsolved” Pakistani subjects may have mutations in novel NL genes not yet identifi from other populations or copy number variants (deletion or insertions) not detected by gene panel sequencing.

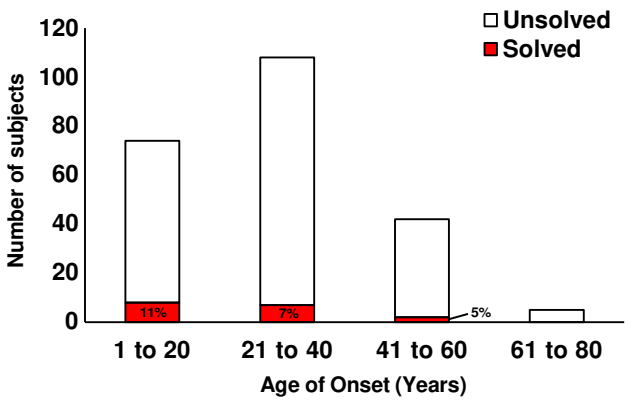

Fig. 3 Distribution of age of onset in NL families in whom a causative mutation was detected in three dominant and one recessive gene. Frequency of subjects solved for causative mutations in one of the known NL genes is binned across ages of onset. Note that genetic diagnoses are most frequent during the first 2 decades of life. Relative frequencies of "solved" versus "unsolved" cases were 1–20 years, 8 of 74 (11%); 21–40 years, 7 of 108 (7%); 41–60 years, 2 of 42 (5%); 61–80 years, 0 of 5 (0%)

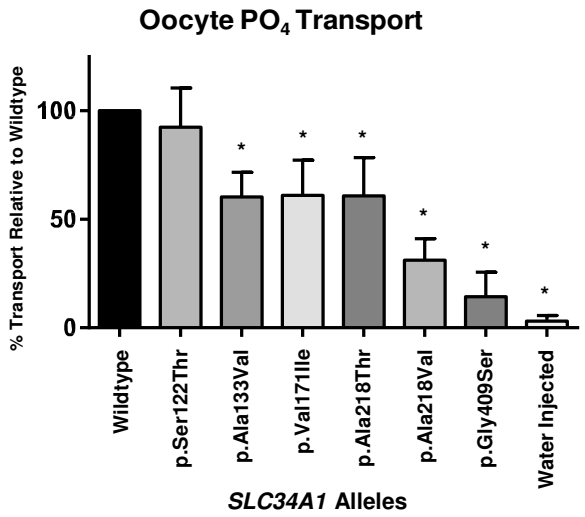

Fig. 4 Effect of Pakistani stone former *SLC34A1* mutations on PO4 transport. Uptake of radioactively labeled phosphate was measured in *Xenopus laevis* oocytes upon injection with *SLC34A1* wildtype and mutant RNA or water as a control. Level of uptake was normalized as a percent of wildtype *SLC34A1*. The above histogram represents the mean of three experiments with standard error of the mean shown by error bars. Asterisk indicates statistically significant difference ($p < 0.05$) from wildtype condition

Alternatively, environmental factors such as chronic dehydration could have a more prevalent role in NL pathogenesis in Pakistan, increasing stone risk suffi y in those lacking a monogenic cause (Rizvi et al. 2002).

The most frequently mutated gene in our cohort was *SLC34A1*, which encodes the proximal tubule sodium–phosphate co-transporter NaPiIIa (Schlingmann et al. 2016). Recessive *SLC34A1* mutations in humans cause infantile

Although more controversial, dominant mutations have been identified in subjects with NL or nephrocalcinosis and

et al. 2016a; Daga et al. 2017; Fearn et al. 2018; Halbritter et al. 2015; Prié et al. 2002; Rajagopal et al. 2014; Schlingmann et al. 2016). Our current study adds 5 novel *SLC34A1* mutations and 12 novel families with dominant *SLC34A1* NL disease to the previous 9 mutations and 10 families in the literature (Braun et al. 2016a; Daga et al. 2017; Fearn et al. 2018; Halbritter et al. 2015; Prié et al. 2002; Rajagopal et al. 2014; Schlingmann et al. 2016). The age of onset did not correlate with mutation severity and, in fact, varied within families with segregating heterozygous *SLC34A1* mutations (Suppl. Figure 6, Table 1). This suggests that other factors (e.g., environmental triggers including diet) likely influence the age-related penetrance of these mutations.

Importantly, the fi e missense mutations in our cohort were functionally validated and harbor defective phosphate uptake activity (Fig. 4) although not through dominant-negative effects on the wildtype protein (Suppl. Figure 8). Studies of *Slc34a1* knockout mice suggest that dominant *SLC34A1* mutations may rather cause stone disease through haploinsufficiency (Beck et al. 1998). Heterozygous null *Slc34a1* knockout mice display abnormalities in calcium/phosphate homeostasis that increase NL risk including increased phosphate excretion and elevated 1,25-$(OH)_2$ vitamin D_3 levels (Prié et al. 2001).

In our previous studies of consanguineous families with NL or other renal disease, we have identifi autosomal recessive mutations in known disease genes in a substantial fraction of cases (Braun et al. 2016a, b; Daga et al. 2017; Halbritter et al. 2015; Sadowski et al. 2015; Warejko et al. 2018). We were, therefore, surprised to identify a recessive cause of NL only once (0.4%) in this cohort despite the high frequency of Pakistani subject consanguinity (Suppl. Table 2). Because of the high degree of consanguinity, NL disease in Pakistani families may exhibit more complex genetic inheritance patterns, as was observed previously with limb–girdle muscular dystrophy on Réunion Island (Beckmann 1996; Richard et al. 1995): single families may have multiple distinct recessive and/or dominant causative mutations, hampering monogenic mutation detection.

Another explanation is that the current cohort and our previous NL studies ascertained different forms of NL disease. The Pakistani NL subjects were uniformly recruited during hospitalization under the care of an expert urologist for a nephrolithiasis episode. In our previous reports, subjects were also recruited by nephrologists in specialty

clinics and, in some cases, presented with broader and more severe clinical features in addition to NL such as failure to thrive, chronic kidney disease, acid–base disturbances, and electrolyte abnormalities (Braun et al. 2016a; Daga et al. 2017; Halbritter et al. 2015). The latter severe cases may, therefore, have distinct genetic etiologies (i.e., mutations in known recessive NL genes) from our Pakistani subjects with isolated NL.

Identifying the monogenic etiology in patients with nephrolithiasis can provide additional preventative, diagnostic, and therapeutic implications in concert with standard clinical diagnostics such as stone analysis and urine metabolite studies that currently guide patient care. For instance, in subject URO-77 bearing a recessive truncation mutation in *APRT* (Table 1, Supplementary Table 4), allopurinol therapy should be initiated to stabilize and/or improve renal function. Despite the declining costs of genetic testing, genetic diagnostics may not be economically feasible for all patients in resource-limited settings such as Pakistan. Until genetic testing becomes more financially viable in these settings, it may be most relevant to cases with severe onset (i.e., early age, renal dysfunction), with strong family history, and where clinical testing does not reveal a precise etiology.

Conclusion

We examined the monogenic causes of NL in a novel geographic cohort and most frequently identifi dominant mutations in the sodium–phosphate transporter SLC34A1 with functional validation.

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Author contributions A.A., A.J.M, I.U., D.B., S.S., A.D., T.J.S., J.A.S., H.Y.G., and J.H. developed and performed gene panel amplifi and massive parallel sequencing, performed variant calling, devised and performed the mutational analysis strategy and application, and conducted assessments of genotype-phenotype correlations. A.A., Ay.A., M.A., and S.K recruited patients and gathered detailed clinical information for the study. T.K., N.H., A.W., and C.W. performed functional *SLC34A1* studies in cell lines and oocytes. All authors critically reviewed the paper. F.H. conceived of and directed the project. A.A., A.J.M, and F.H. wrote the paper.

Compliance with ethical standards

Conflict of interest F.H. is a co-founder of Goldfinch Biopharma Inc. The other authors declare that they have no competing financial interests. No part of this manuscript has been previously published.

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Supplementary Methods 1

Preparation of Plasmid Constructs/Mutagenesis

To study the expression of wildtype and mutant *SLC34A1* in mammalian cells, *SLC34A1* constructs were fused at the c-terminus to *EGFP* by subcloning the cDNAs into the pEGFP-C1 vector (Clontech) or *RFP* (kindly provided by Andreas Werner). Missense mutations identified in Pakistani stone formers (**Table 1**) were introduced into *SLC34A1-RFP* constructs by site-directed mutagenesis using the QuickChange II kit (Agilent). Mutagenic primers were designed using the Agilent web based QuickChange® Primer Design software Program. XL1-Blue ultracompetent cells were transformed with the PCR products from the site-directed mutagenesis. The plasmids containing the generated mutations were isolated using QIAprep Spin Miniprep (Qiagen, 27106) and all mutations were confirmed by sequencing (Microsynth). One clone from each construct was used for the experiments.

PCR templates used for cRNA synthesis were generated by specific primers introducing T3 promoter sequence upstream of the starting ATG of *SLC34A1*, *EGFP* or *RFP* respectively at the 5' end and a 3' primer maintaining the SV40 polyA terminator signal.

Primers	**5'-3'**
T3 hNaPi-IIa:	agccacAATTAACCCTCACTAAAGGGAGACCACTCCACCCTCGAGATGttgtcctacggagag
T3 RFP-hNaPi-IIa:	agccacAATTAACCCTCACTAAAGGGAGATACCGGTGATATACATATGCGGGGTTCTCATCATC
T3 EGFP-hNaPi-IIa:	agccacAATTAACCCTCACTAAAGGGAGAGCTACCGGTCGCCACCATGGTGAGCAAGGGCGAG
SV40 pa terminator	TTTTTTTTTTTTTTTTTTTTTTTTTTTCAAAATATTAACGCTTACAATTTACGCG

Sequence of PCR products were confirmed by sequencing (Microsynth). For heterologous expression in Xenopus laevis oocytes, capped cRNA was synthesized in vitro using Megascript T3 kit (Ambion) in the presence of cap analog (New England Biolabs).

Cell culture and transient transfections

Opossum kidney cells (clone 3B/2) were cultured in DMEM / Ham's F-12 medium (1:1) supplemented with 10% fetal calf serum, 2 mM glutamine, 20 mM HEPES and 50 IU/ml penicillin/streptomycin. Cells were plated on coverslips in 12 multiwell plates (TPP), and cultures were transfected with either wildtype *SLC34A1-EGFP* and wildtype or

mutant *SLC34A1-RFP*. Cultures were transfected at 70% confluency by an overnight incubation in 500 µl OPTIMEM (GIBCO, 31985-047) containing 1 µg of DNA and 3 µl of Lipofectamine™ 2000 Reagent (Invitrogen, 11668-019). We performed two independent experiments, each in duplicates or triplicates.

Actin staining and confocal microscopy

Upon expression of clear patches of wildtype *SLC34A1* signal (2-3 days after transfection), cells were fixed and permeabilized with saponin. Thereafter, actin was stained by incubation with Alexa Fluor™ 647 Phalloidin (Invitrogen) diluted 1:500. After incubation for 30 min in the dark, cells were washed three times with PBS/saponin and once with PBS. The coverslips were then mounted on microscope slides using Dako Glycergel® Mounting Medium. The subcellular final locations of the transfected cotransporters were analyzed by Confocal Laser Scanning Microscopy (Leica SP2) using a 63X oil immersion objective at the Center for Microscopy and Image Analysis at the University of Zurich.

Supplementary Table 1. Thirty genes that are known to cause monogenic forms of NL/NC and that were included in this study.

	Gene Symbol	Gene Name	Accession #	Disease entity	MIM Phenotype #	Mode	Coding Exons	Ref.
1	***AGXT***	Alanine-glyoxylate aminotransferase	NM_000030.2	Primary hyperoxaluria, type 1	259900	AR	11	1
2	***APRT***	Adenine phosphoribosyltransferase	NM_000485.2	Adenine phosphoribosyltransferase deficiency, Urolithiasis (DHA stones), renal failure	614723	AR	5	2
3	***ATP6V0A4***	ATPase, H+ transporting, lysosomal V0 subunit a4	NM_020632.2	dRTA	602722	AR	20	3
4	***ATP6V1B1***	ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B1	NM_001692.3	Distal renal tubular acidosis (dRTA) with deafness	267300	AR	14	4
5	***CA2***	Carbonic anhydrase II	NM_000067.2	Osteopetrosis + d/pRTA	259730	AR	7	5
6	***CASR***	Calcium-sensing receptor	NM_001178065.1	Hypocalcemia with Bartter syndrome / hypocalcemia, autosomal dominant	601198	AD	6	6
7	***CLCN5***	Chloride channel, voltage-sensitive 5	NM_001127898.3	Dent disease / Nephrolithiasis, type 1	300009 / 310468	XR	14	7
8	***CLCNKB***	Chloride channel, voltage-sensitive Kb	NM_000085.4	Bartter syndrome, type 3	607364	AR	19	8
9	***CLDN16***	Claudin 16	NM_006580.3	Familial hypomagnesemia with hypercalciuria & nephrocalcinosis (FHHNC)	248250	AR	5	9
10	***CLDN19***	Claudin 19	NM_001123395.1	Familial hypomagnesemia with hypercalciuria & nephrocalcinosis (FHHNC) with ocular abnormalities	248190	AR	4	10
11	***CYP24A1***	Cytochrome P450, family 24, subfamily A, polypeptide 1	NM_000782.4	1,25-(OH) vitamin D-24 hydroxylase deficiency, infantile hypercalcemia	143880	AR	11	11
12	***FAM20A***	Family with sequence similarity 20, member A	NM_017565.3	Enamel-renal syndrome, amelogenesis imperfect and nephrocalcinosis	204690	AR	12	12
13	***GRHPR***	Glyoxylate reductase/hydroxypyruvate reductase	NM_012203.1	Primary hyperoxaluria, type 2	260000	AR	9	13
14	***HNF4A***	Hepatocyte nuclear factor 4, alpha	NM_000457.4	Mature onset diabetes of the young Fanconi syndrome + Nephrocalcinosis	125850	AD	1	14
15	***HOGA1***	4-hydroxy-2-oxoglutarate aldolase 1	NM_138413.3	Primary hyperoxaluria, type 3	613616	AR	7	15
16	***HPRT1***	Hypoxanthine phosphoribosyltransferase 1	NM_000194.2	Kelley-Seegmiller syndrome, partial HPRT deficiency, HPRT-related gout	300323	XR	9	16

17	***KCNJ1***	Potassium inwardly-rectifying channel, subfamily J, member 1	NM_000220.4	Bartter syndrome, type 2	241200	AR	2	17
18	***OCRL***	Oculocerebrorenal syndrome of Lowe	NM_000276.3	Lowe syndrome / Dent disease 2	309000 / 300555	XR	24	18
19	***SLC12A1***	Solute carrier family 12, member 1	NM_000338.2	Bartter syndrome, type 1	601678	AR	27	19
20	***SLC22A12***	Solute carrier family 22 (organic anion/urate transporter), member 12	NM_144585.3	Renal hypouricemia, RHUC1	220150	AR/AD	10	20
21	***SLC26A1***	Solute carrier family 26 (sulfate transporter), member 1	NM_213613	Nephrolithiasis, calcium oxalate	167030	AR	4	21
22	***SLC2A9***	Solute carrier family 2 (facilitated glucose transporter), member 9	NM_001001290.1	Renal hypouricemia, RHUC2	612076	AR/AD	13	22
23	***SLC34A1***	Solute carrier family 34 (sodium phosphate), member 1	NM_003052.4	Hypophosphatemic nephrolithiasis/osteoporosis-1, NPHLOP1 / Fanconi renotubular syndrome 2	612286 / 613388	AR/AD	13	23
24	***SLC34A3***	Solute carrier family 34 (sodium phosphate), member 3	NM_001177316.1	Hypophosphatemic rickets with hypercalciuria	241530	AR	12	24
25	***SLC3A1***	Solute carrier family 3 (cystine, dibasic and neutral amino acid transporters, activator of cystine, dibasic and neutral amino acid transport), member 1	NM_000341.3	Cystinuria, type A	220100	AR	10	25
26	***SLC4A1***	Solute carrier family 4, anion exchanger, member 1 (erythrocyte membrane protein band 3, Diego blood group)	NM_000342.3	Primary distal renal tubular acidosis, dominant / recessive	179800 / 611590	AR/AD	19	26
27	***SLC7A9***	Solute carrier family 7 (glycoprotein associated amino acid transporter light chain, bo,+ system), member 9	NM_014270.4	Cystinuria, type B	220100	AR/AD	12	27
28	***SLC9A3R1***	Solute carrier family 9, subfamily A (NHE3, cation proton antiporter 3), member 3 regulator 1	NM_004252.4	Hypophosphatemic nephrolithiasis/osteoporosis 2, (NPHLOP2)	612287	AD	6	28
29	***VDR***	Vitamin D (1,25- dihydroxyvitamin D3) receptor	NM_000376.2	Idiopathic hypercalciuria	277440	AD	11	29
30	***XDH***	Xanthine dehydrogenase	NM_000379.3	Xanthinuria, type 1	278300	AR	36	30

AD, autosomal dominant; AR, autosomal recessive.

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Supplementary Table 2A. Mutation detection frequency *versus* stone recurrence status in probands of 229 NL families*.

	Detection of causative mutation		
Subject with stone recurrence	**Yes (% Total)**	**No**	**Total**
Yes	8 (8%)	105	113
No	9 (8%)	107	116
Total	17	212	**229**

Odds Ratio 0.91 (95% CI 0.34-2.4), p = 0.84
*No data for 6 families.

Supplementary Table 2B. Mutation detection frequency *versus* positive family history in 229 NL families*.

	Detection of causative mutation		
Positive Family History	**Yes (% Total)**	**No**	**Total**
Yes	13 (12%)	96	109
No	4 (3%)	116	120
Total	17	212	**229**

Odds Ratio 3.9 (95% CI 1.2-12.4), p = 0.02
*No data for 6 families.

Supplementary Table 2C. Mutation detection frequency *versus* reported consanguinity status in 229 NL families*.

	Detection of causative mutation		
Subject with reported consanguinity	**Yes (% Total)**	**No**	**Total**
Yes	10 (8%)	112	122
No	7 (7%)	100	107
Total	17	212	229

Odds Ratio 1.28 (95% CI 0.47-3.5), p = 0.6344
*No data for 6 families.

Supplementary Table 3. Variant of unknown significance in *SLC34A1* in 1 family (0.4%) out of 235 families with NL from Punjab province in Pakistan.

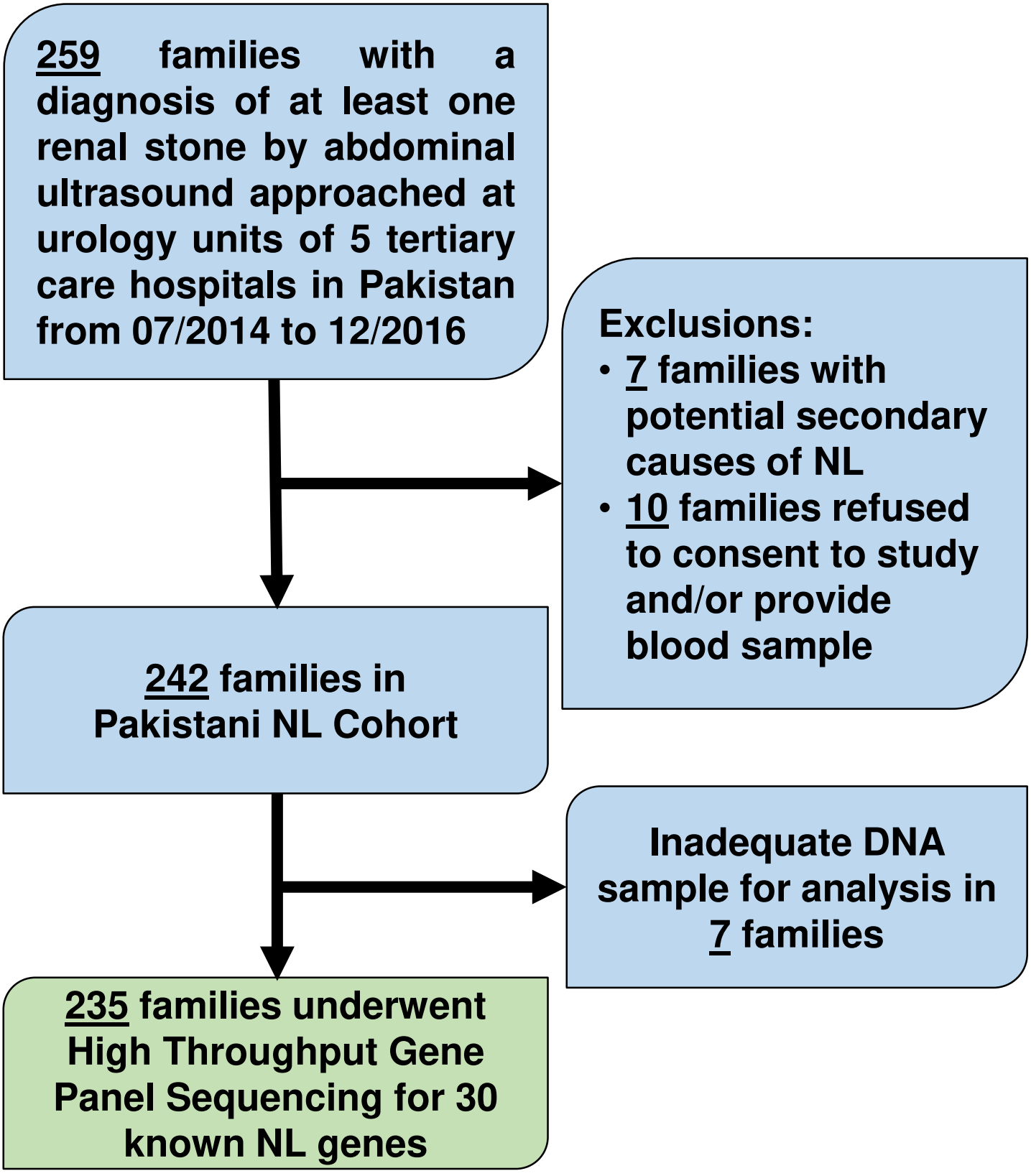
Family_ Individual	Nucleotide change	Amino acid change	Zygosity State	PPh2, Evolutionary Conservation	gnomAD Allele Frequency*	Ref.	Sex	Age of onset (years)	Stone recurrence	Family history	Parental consanguinity	Phenotype prior to mutational analysis
***SLC34A1* [Type 2 sodium/phosphate cotransporter]; Nephrolithiasis/osteoporosis, hypophosphatemic, 1**												
URO-131	c.365G>C	p.Ser122Thr	het	0.965, *C.e.*	NP	Novel	M	45	Yes	N	Y	NL

C.e., Caenorhabditis elegans; het, heterozygous; M, male; N, no; NA, not applicable; NL, nephrolithiasis; NP, not present; PPh2, Polyphen2-HumVar (http://genetics.bwh.harvard.edu/pph2/); Y, yes. *Frequency reported as "number of homozygotes/number of variant alleles/total alleles" in Genome Aggregation Database (gnomAD).

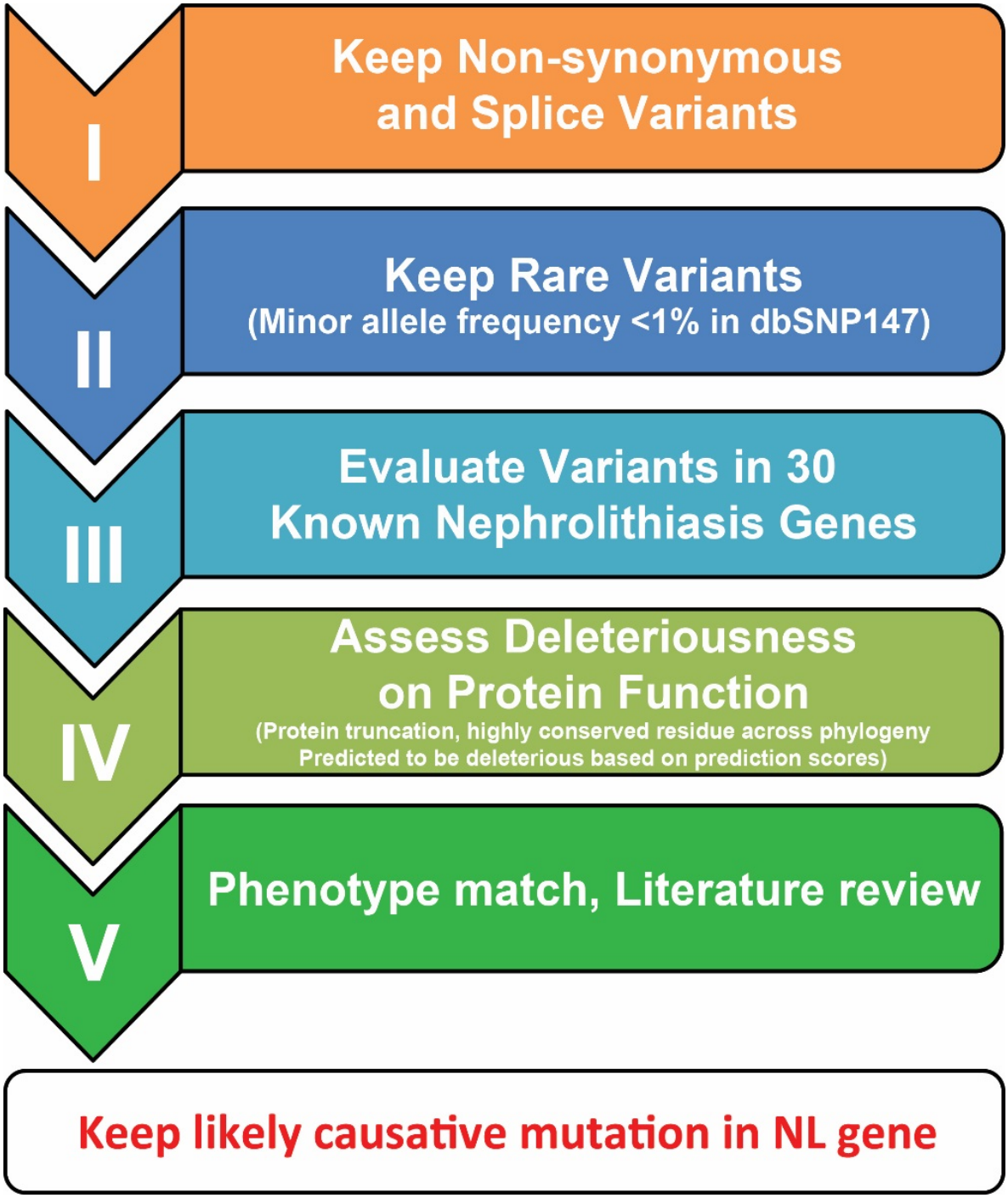
Supplemental Table 2: Clinical implications following detection of a monogenic cause of NL/NC.

Gene (Family ID)	Clinical Implications Based on Gene-Specific Associated Phenotypes
SLC34A1 UF26 UF30 URO-11 URO-12 URO-13 URO-17 URO-29 URO-88 URO-117 URO-131 URO-133 URO-140 URO-169	1. Genetic counseling 2. Screen for bone mineralization disease.
SLC4A1 URO-35 URO-178 URO-196	1. Genetic counseling 2. Screen for hemolytic anemia 3. Screen for urine acidification defects 4. Screen for acidemia, hypokalemia, and hypocalcemia. 5. Screen for bone mineralization disease.
SLC7A9 URO-152	1. Genetic counseling 2. Measure urine cystine levels 3. Consider alkalinization and pencillamine therapies.
APRT URO-77	1. Genetic counseling 2. Allopurinol therapy

NC, nephrocalcinosis; NL, nephrolithiasis.



Supplementary Figure 1. Patient recruitment and sequencing workflow. Genomic DNA from 235 families (350 affected patients) with a clinical diagnosis of NL were sequenced for causative mutations in 30 known genes that cause NL, using barcoded multiplex chain reactions and next generation sequencing approach[4,5].



Supplementary Figure 2. Schematic view of the variant filtering pipeline implemented for the identification of known gene mutations resulting in NL phenotype.

I. Keep non-synonymous variants and intronic variants that are located within splice sites.

II. Keep rare variants present with minor allele frequency (MAF) <1% in healthy control cohorts dbSNP147 (https://www.ncbi.nlm.nih.gov/projects/SNP).

III. Applying known gene approach by selecting all variants detected in known nephrolithiasis genes (**Suppl. Table 1**).

IV. Ranking of remaining variants based on their predicted likelihood to be deleterious for the function of the encoded protein using **Polyphen 2** (http://genetics.bwh.harvard.edu/pph2, **SIFT** (http://sift.jcvi.org/) and **Mutation Taster** (http://www.mutationtaster.org)

V. Reviewing literature and review with referring physician delineating whether the detected mutation matches the phenotype.

Autosomal recessive variants

Include homozygous or compound heterozygous alleles as disease causing if:

- Truncating mutation (stop, abrogation of start or stop, obligatory splice, frameshift) **or**
- Missense mutation if any of following criteria were met:
  - Predicted deleterious for the protein function (at least in two among three prediction programs (Polyphen (>0.5), SIFT (Del.), Mutation taster (D.C.))
  - Continuously conserved at least among vertebrates (or beyond)
  - Previously reported as disease causing with functional evidence implicating mutation causality

Exclude allele as disease causing if:

- Allele frequency
  - Allele frequency >1% (in EVS server, ExAC, gnomAD, 1000 genomes)
- Non segregation
  - if compound heterozygous variants are in cis or if an affected family member is without the variant or an unaffected family members is with the variant

Discussion of genotype-phenotype correlation in a panel of nephro-geneticists followed by review of clinical phenotype with referring physician

Autosomal dominant variants

Include heterozygous alleles as disease causing if:

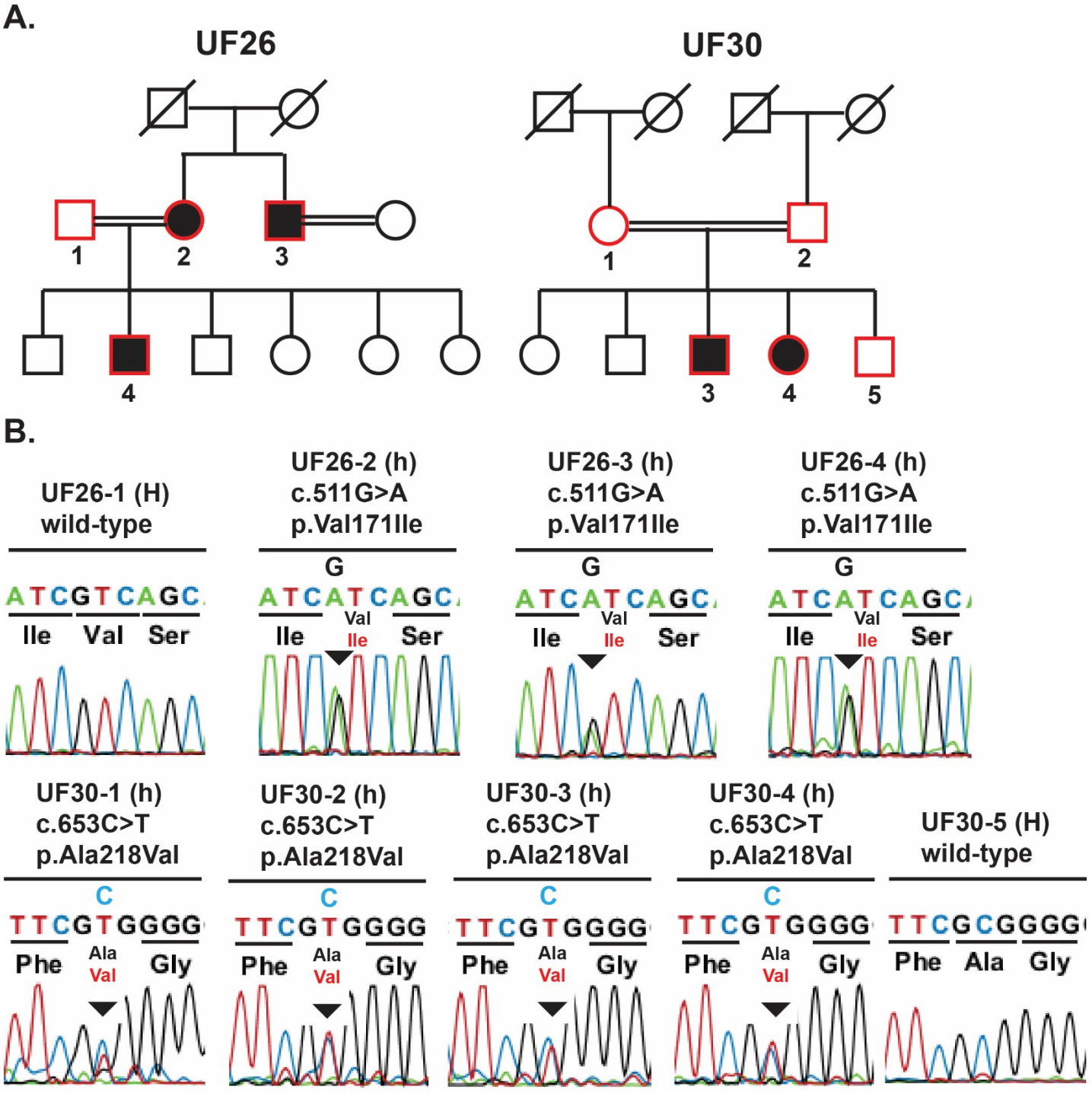
- Truncating mutation (stop, abrogation of start or stop, obligatory splice, frameshift) **or**
- Missense mutation if any of following criteria were met:
  - Predicted deleterious for the protein function (at least in two among three prediction programs (Polyphen (>0.5), SIFT (Del.), Mutation taster (D.C.))
  - Continuously conserved at least among vertebrates (or beyond)
  - Previously reported as disease causing with functional evidence implicating mutation causality

Exclude allele as disease causing if:

- Allele Frequency
  - Allele frequency >0.1% (in EVS server, ExAC, gnomAD, 1000 genomes) unless functional data exists to support the variant
- Non segregation
  - if an affected family member is without the variant
- If an unaffected family member is with the allele consider incomplete penetrance and variable expressivity

Discussion of genotype-phenotype correlation in a panel of nephro-geneticists followed by review of clinical phenotype with referring physician

Supplementary Figure 3. Variant Criteria for Autosomal Recessive and Dominant Variant Analysis



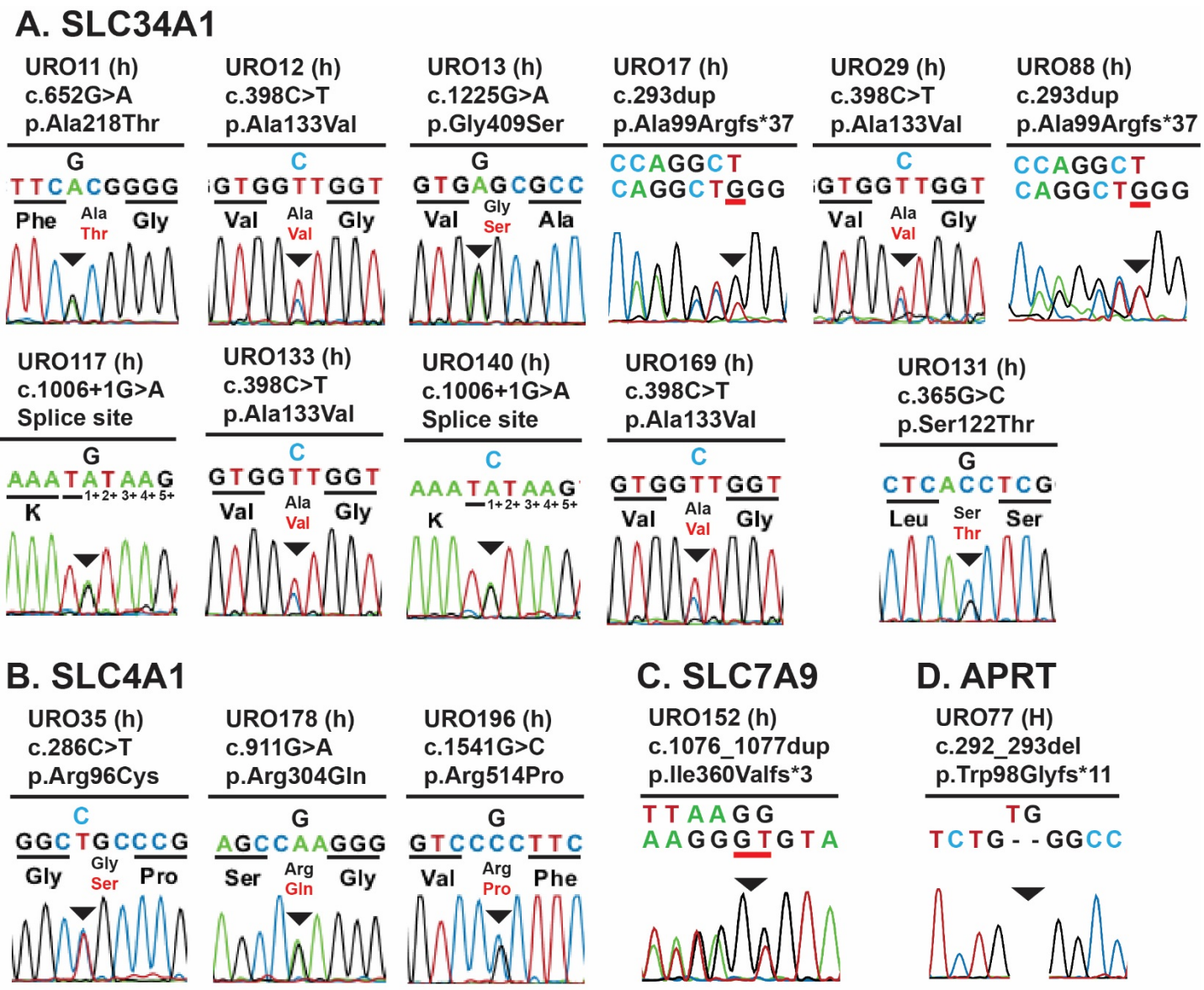
Supplementary Figure 4. Pedigree and DNA sequencing confirmation of mutations in NL families.

A. Pedigree structures for two families UF26 and UF30 with *SLC34A1* mutations.

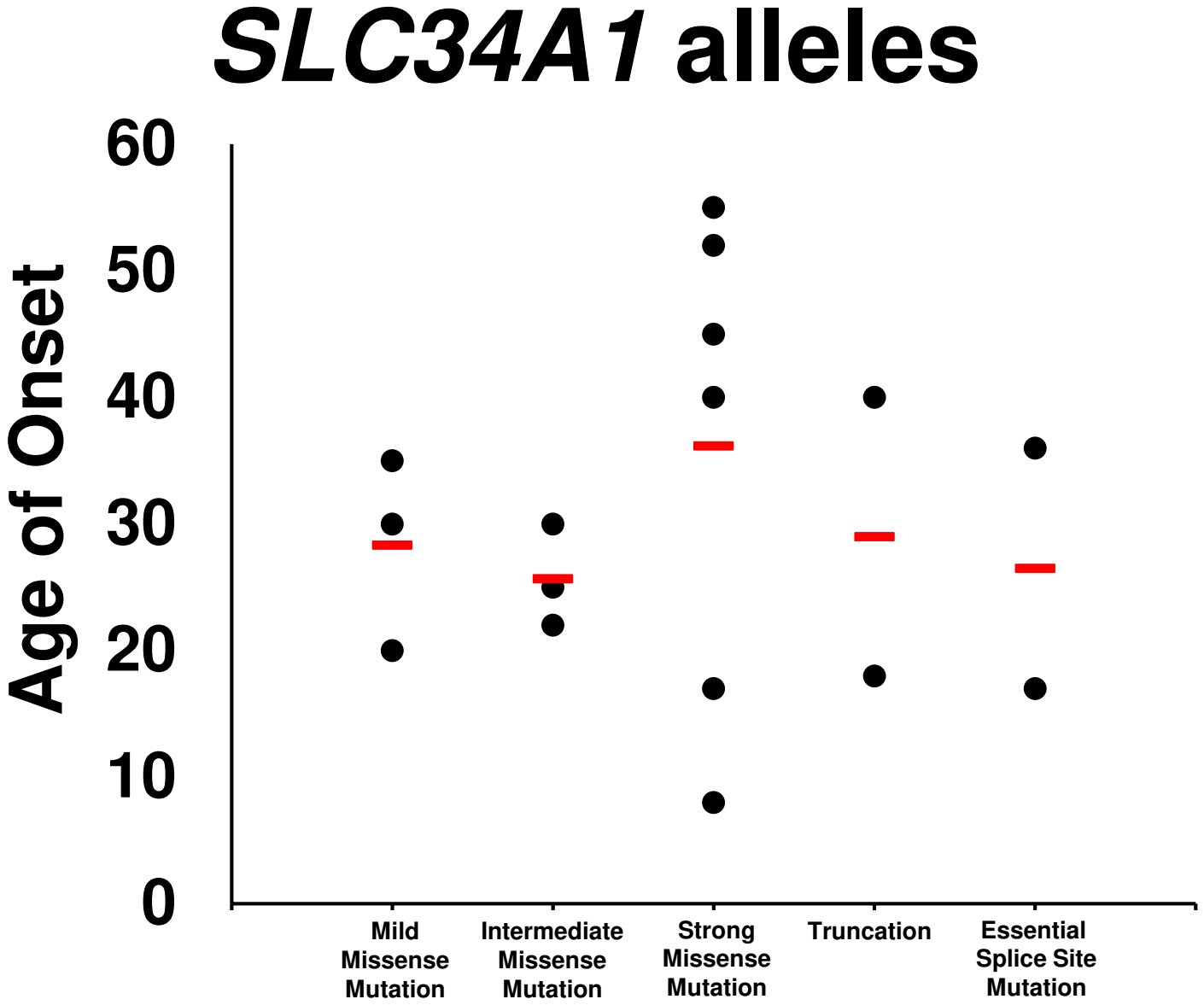
B. Sanger sequencing tracing of *SLC34A1* mutations in subjects.

Family numbers are shown on top, and cDNA change (c.) and protein sequence change (p.) are shown below. Arrow heads indicate position of mutated nucleotide.

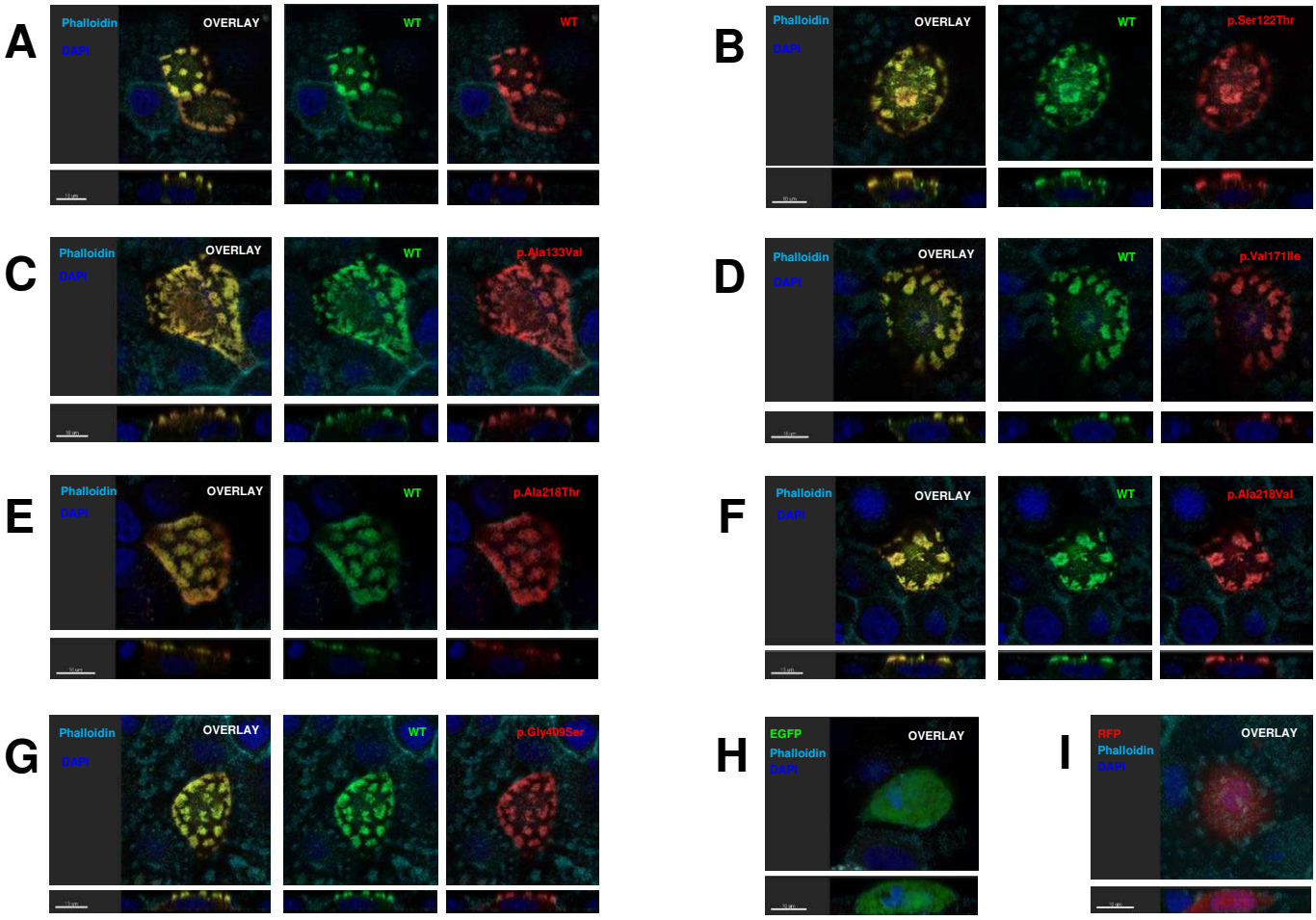
H, homozygous; h, heterozygous.



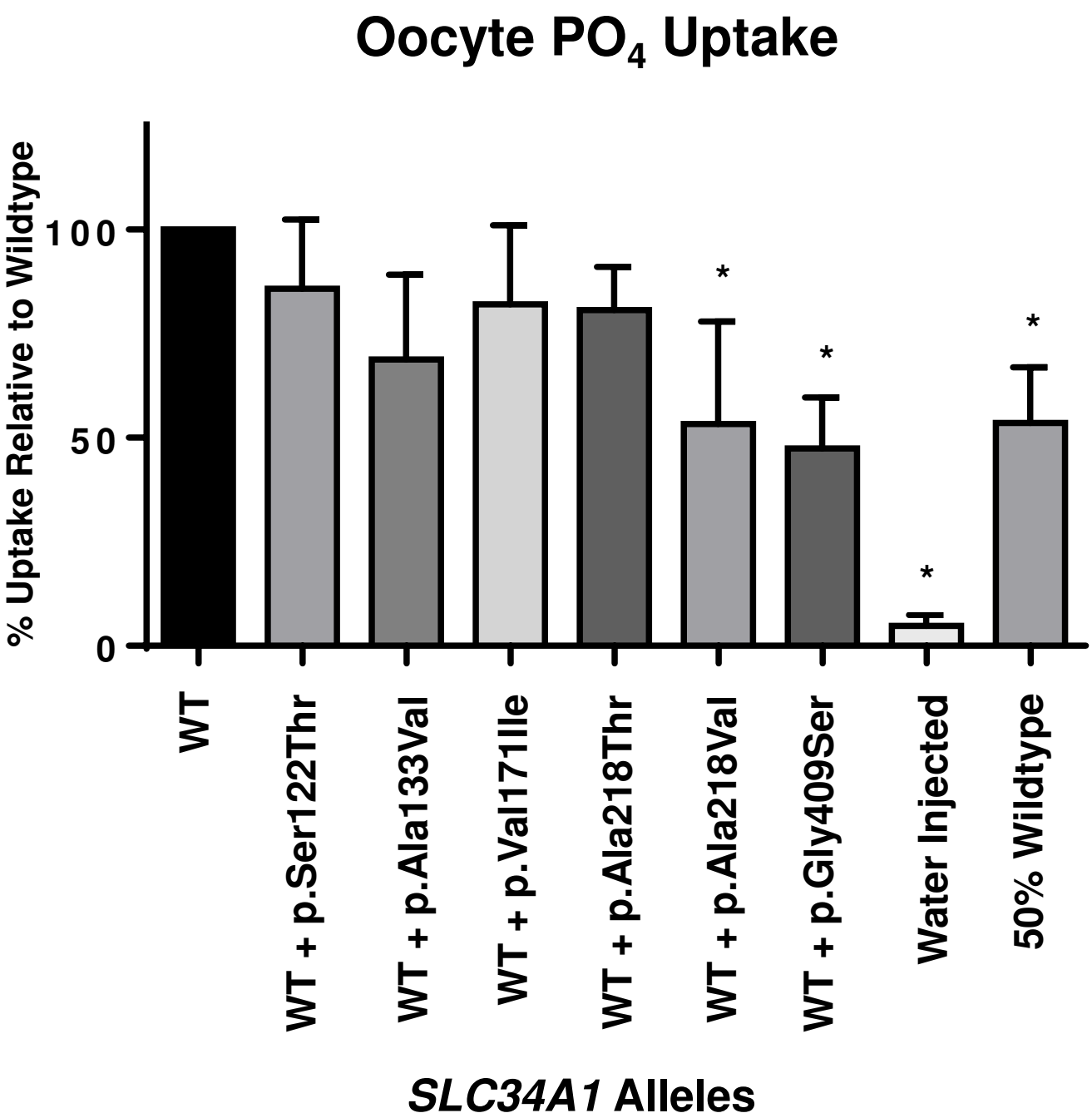
Supplementary Figure 5. NA sequencing confirmation of mutations in NL singlets.
A. Sanger traces of *SLC34A1* mutations in families URO11, URO12, URO13, URO17, URO29, URO88, URO117,URO133, URO140, and URO169. Sanger trace of *SLC34A1* variant of unknown significance in URO131 is also shown.
B. Sanger traces of *SLC4A1* mutations in subjects URO35, URO 178 and URO196.
C. Sanger traces of *SLC7A9* mutations in subject URO152.
D. Sanger traces of *APRT* mutations in subject URO77.
Family numbers are shown on top, and cDNA change (c.) and protein sequence change (p.) are shown below. Arrow heads indicate position of mutated nucleotide.
(H), homozygous; (h), heterozygous.



Supplementary Figure 6. Genotype-Phenotype Correlation between Dominant SLC34A1 Alleles and Age of Onset in Pakistani Stone Formers. Mutations were grouped by effect on protein coding and plotted versus age of onset. Red bars indicate mean age of onset for each group with no trend noted. Mild missense mutations achieved our minimal inclusion criteria, intermediate missense mutations additionally had conservation through invertebrates, and strong missense mutations showed strong conservation through invertebrates and PolyPhen-2 score >0.9.



Supplementary Figure 7. Co-expression and plasma membrane localization of *SLC34A1* wild-type and mutated constructs. In panels A-G, EGFP-tagged WT *SLC34A1* (encoding NaPiIIa) was co-expressed with RFP-tagged *SLC34A1* wild-type or mutated constructs based on Pakistani stone family mutations in Opossum kidney cells. In A-G, EGFP- and RFP-tagged *SLC34A1* constructs showed appropriate trafficking and co-localization to the cell surface. In H and I, EGFP and RFP mock control constructs showed appropriate cytoplasmic localization.



Supplemental Figure 8. Effect of Pakistani stone former *SLC34A1* mutations on PO4 transport upon co-expression with wildtype *SLC34A1*. Uptake of radioactively labeled phosphate was measured in *Xenopus laevis* oocytes upon injection with either *SLC34A1* wild-type (WT) RNA, 1:1 ratio of WT and mutated *SLC34A1* RNA and mutant constructs, 50% wild-type *SLC34A1* RNA or water as a control. Level of uptake was normalized as a percent of wild-type *SLC34A1*. The above histogram represents the mean of 3 experiments with standard error of the mean shown by error bars.
*Statistically significant difference ($p<0.05$) from Wildtype condition.